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Metabolism of 4-*N*-Hydroxy-Cytidine in *Escherichia coli*

R. B. TRIMBLE AND FRANK MALEY

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

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4-*N*-hydroxy-cytidine was found to substitute for uridine as a pyrimidine supplement for the growth of *Escherichia coli* Bu⁻. Measurement of the incorporation of 4-*N*-hydroxy-cytidine-2-^{14}C into ribonucleic acid and deoxyribonucleic acid revealed that this compound was converted to cytidine or uridine before utilization. Two pathways for metabolism were considered: (i) the reduction of 4-*N*-hydroxy-cytidine to cytidine followed by deamination, (ii) the direct hydrolysis of hydroxylamine from 4-*N*-hydroxy-cytidine to yield uridine. A threefold increase in cytidine (deoxycytidine) deaminase (EC 3.5.4.5) activity, when the cells were grown on 4-*N*-hydroxy-cytidine, suggested the involvement of this enzyme. More direct proof was obtained by purifying the deaminase 185-fold and finding that it released hydroxylamine from 4-*N*-hydroxy-cytidine at one-fiftieth the rate at which ammonia was removed from cytidine. This result is consistent with the slower rate of growth of the Bu⁻ cells on 4-*N*-hydroxy-cytidine than cytidine and suggests that the second pathway is the major route for utilization of this compound.

The mutagenicity of hydroxylamine is believed to result from its reaction with deoxyribonucleic acid (DNA) cytosine, leading to a GC → AT transition (10, 11). By utilizing this selective reaction of hydroxylamine, Brown and Schell (4) were able to synthesize the 4-*N*-hydroxy derivatives of cytosine, cytidine, and deoxycytidine. The 4-*N*-hydroxy-deoxycytidylate (4-*N*-HO-dCMP) analogue of deoxycytidylate was shown to be an effective inhibitor of both thymidylate synthetase (EC 2.1.1.b; reference 22, 25) and deoxycytidylate deaminase (EC 3.5.4.12; reference 23), suggesting that 4-*N*-hydroxy-cytidine (4-*N*-HO-Cyd) derivatives might substitute for the pyrimidine nucleotides at the polynucleotide level. Although labeled 4-*N*-HO-dCMP was incorporated into chick embryo DNA (22), it was present only as deoxycytidylate and deoxythymidylate. These studies suggested that the 4-*N*-HO-dCMP was reduced to deoxycytidylate before utilization.

Because the metabolism of 4-*N*-hydroxy-pyrimidines in animal tissues is rather poorly defined, mainly as a consequence of the low level at which this occurs, it was hoped that a bacterial system might clarify the mechanisms involved.

MATERIALS AND METHODS

Bacterial cultures, media, and growth conditions. Bacterial strains used were *Escherichia coli* B and the pyrimidine-requiring strains Bu⁻ (6) and OK 305. The latter has a reduced capacity to deaminate cytidine and deoxycytidine (17). *E. coli* B was maintained on nutrient agar (Difco), and Bu⁻ and OK 305 were maintained on M9 (1) agar containing 0.2% glucose and 20 μg of uracil per ml. The pyrimidine-requiring strains were checked periodically for purity by plating on unsupplemented M9 agar containing 0.2% glucose. Growth studies with Bu⁻ and OK 305 were conducted at 37 C in optically matched cotton-plugged tubes (18 by 150 mm) containing 6.1 ml of M9 medium plus 0.4% glucose and 0.065 mM of the pyrimidine supplement. These were inoculated with 0.2 ml of a culture grown to stationary phase in M9 medium plus 0.2% glucose and a 0.1 mM supplement of uracil or 4-*N*-HO-Cyd as indicated. The extent of growth was followed by the absorbance at 650 nm with a spectrophotometer (Coleman, Jr., model II). Optical density readings were converted to cell titers with a standard curve prepared against a total count of the appropriate organism. For larger cell yields, liter cultures of the desired organism were grown in M9 medium plus 0.4% glucose and 0.1 mM pyrimidine supplement where required. After growth of the cells to late log phase (5×10^8 bacteria/ml) at 37 C with vigorous aeration on a rotary shaker, they were chilled to 0 C, harvested by centrifugation, and washed twice in the cold with 0.9% NaCl. The final cell pellet was used immediately for the extraction of nucleic acids or the preparation of cell-free extracts described below.

Materials. Cytidine-2-^{14}C (specific activity, 21 mCi/mmole) and enzyme-grade ammonium sulfate were purchased from Schwarz Mann Co., Orangeburg, N.Y.; pyrimidine nucleosides and bases and reduced nicotinamide adenine dinucleotide phosphate

(NADPH) from Sigma Chemical Co., St. Louis, Mo.; streptomycin sulfate from Nutritional Biochemicals Corp., Cleveland, Ohio; diethylaminoethyl (DEAE) cellulose (1 meq/g) from Schleicher & Schuell Co., Keene, N.H.; alkaline phosphatase (EC 3.1.3.1), phosphodiesterase (EC 3.1.4.1), and DNase I (EC 3.1.4.5) from Worthington Biochemical Corp., Freehold, N.J.; and hydroxylammonium acetate from Eastman Organic Chemicals, Rochester, N.Y. All other chemicals and solvents were reagent grade or the purest grade available.

4-*N*-HO-Cyd and 4-*N*-hydroxy-deoxycytidine (4-*N*-HO-dCyd) were prepared essentially by the method of Brown and Schell (4), except that, after acid rearrangement, the reaction solution was neutralized with Dowex 1-CO_3 and passed through a column of Dowex 50^+H (200 to 400 mesh), and the desired compounds were eluted with 3% NH_4OH. Radioactive 4-*N*-HO-Cyd-2-^{14}C was prepared by a small-scale modification of the above procedure in which 100 μmoles of unlabeled cytidine was added to 50 μCi of cytidine-2-^{14}C in 5.0 ml, followed by 2.35 g of solid hydroxylammonium acetate to give a final concentration of 5 N at a *p*H of 6.0. The 4-*N*-hydroxy derivatives had absorption spectra at *p*H 2.1 and 7.5, identical with those reported by Fox et al. (9). The cytidine derivative migrated as a homogeneous spot on paper chromatograms developed in solvent A, with an R_F of 0.35 compared with an R_F of 0.26 for cytidine. In the case of 4-*N*-HO-Cyd-2-^{14}C, the radioactivity corresponded to the ultraviolet absorbing area. Paper chromatograms of 4-*N*-HO-dCyd developed in solvent A revealed the major component to migrate with an R_F of 0.52 and a trace component that migrated with deoxycytidine at an R_F of 0.40.

Tetrahydrouridine was prepared by the reduction of cytidine as described by Hanze (13).

Extraction of RNA and DNA. Nucleic acids were extracted from 0.5 g (wet weight) of cells by a modified Schmidt-Thanhauser procedure (14). The ribonucleic acid (RNA) hydrolysate was neutralized with NaOH, and the nucleotides were separated on Dowex 1-formate (22). When guanosine monophosphate did not separate completely from uridine monophosphate, the peak area of the latter was lyophilized and taken up in 0.2 ml of water, and the following components were added: 0.03 ml of 1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.5), 0.05 ml of 0.1 M $MgCl_2$, and 0.1 ml of alkaline phosphatase (0.5 mg/ml). The mixture was incubated for 6 hr at 37 C, and samples were spotted on paper chromatograms which were developed in solvent system C.

DNA was hydrolyzed enzymatically to the 5'-mononucleotides and separated by paper electrophoresis (22).

Preparation of cell-free extracts. Cell-free extracts were prepared at 0 to 4 C by sonically oscillating 5.0 ml of a 10% suspension (wet weight/volume) of washed cells in 0.05 M Tris-hydrochloride or potassium phosphate buffer (*p*H 7.1) for 2 min (Biosonik II, microprobe), followed by centrifugation at 30,000 × *g* for 30 min. Crude extracts routinely contained 8 to 11 mg of protein per ml.

Enzyme assays: assay I. Deamination was determined in reaction mixtures containing cytidine-2-^{14}C, 0.25 μmole (2.1×10^6 counts per min per μmole); Tris-hydrochloride or potassium phosphate (*p*H 7.1), 20 μmoles; cell-free extract, 0.02 to 0.05 ml; and water to a final volume of 0.3 ml. The reactions were terminated after 10 min of incubation at 37 C by heating at 100 C for 2 min, diluting to 2.0 ml with water, and removing the denatured protein by centrifugation. The same procedure was used with 2.0 μmoles of 4-*N*-HO-Cyd-2-^{14}C (9.1×10^5 counts per min per μmole) as substrate, except that the reaction volume was increased to 0.5 ml to accommodate 0.2 to 0.3 ml of cell-free extract, and the incubation time was extended to 30 or 60 min.

The supernatant fractions were passed through mixed resin columns consisting of Dowex 1-formate over Dowex 50^+H (50 to 100 mesh; 1 by 3 cm each). The precipitates were washed with an additional 2.0 ml of water and centrifuged, and the supernatant fractions were again added to the column. The columns were washed with water until 20 ml was collected, and 0.5-ml samples were measured for radioactivity in a scintillation counter. These values were corrected by subtracting an identically treated zero-time control. The remaining column eluates were lyophilized, taken up in 0.05 ml of water, and chromatographed in solvent A. One unit of activity is defined as the deamination of 1 μmole of cytidine per min under the above reaction conditions.

Assay II. 4-*N*-HO-Cyd conversion to uridine or uracil was determined also by measuring the change in absorbancy at 290 nm. Reaction mixtures containing substrate, 1.0 μmole; potassium phosphate (*p*H 7.1), 30 μmoles; cell-free extract, 0.2 to 0.3 ml; and water to a final volume of 0.5 ml were set up in duplicate, with one serving as a substrate-free control. After incubation at 37 C for various times, reactions were terminated by the addition of 0.5 ml of ice-cold 0.6 N perchloric acid, and the precipitated protein was removed by centrifugation. A 0.2-ml portion of the supernatant fraction was diluted to 2.0 ml with water, and the absorbancy of this solution was determined at 290 nm in a spectrophotometer (Gilford model 2400). The absorbancy of similarly treated substrate-free controls was subtracted from the test sample, and the resultant value was subtracted from a pair of identically treated zero-time controls. The formation of product in micromoles was determined from a measured millimolar absorbancy change of 8.95 for uracil and 8.59 for uridine (2, 9).

For stoichiometry studies on the conversion of 4-*N*-HO-Cyd to products by purified enzyme preparations, assay II was modified as follows. The reaction volume was increased to 4.8 ml and contained substrate, 17 μmoles; potassium phosphate (*p*H 7.1), 100 μmoles; purified enzyme protein, 1.2 mg; and water to volume. The reaction was started by addition of enzyme; at various times, 0.5-ml portions were transferred to a pair of tubes at 0 C. One contained 0.5 ml of 0.6 N perchloric acid and was used for the determination of nucleoside, hydroxylamine (8), and ninhydrin-positive material (7). The other contained 0.5 ml of 1 M trichloroacetic acid and was used for the determination of ammonia (30).

Chemical assays for hydroxylamine, ninhydrin-positive material, and ammonia are described in detail below. All assays were compared with identically treated zero-time controls.

Assay III. To follow reaction velocities and to locate enzyme activity during purification, a kinetic assay was used to measure the decrease in absorbancy at 290 nm with time (24). For these measurements, a spectrophotometer (Gilford model 2400) was employed. A routine assay mixture contained 0.4 to 0.5 μmole of substrate; potassium phosphate (*p*H 7.1), 20 μmoles; and enzyme and water to 1.0 ml. The decrease in absorbancy at 30 C was recorded automatically after the addition of enzyme. The amount of substrate converted to product (uridine or deoxyuridine) in micromoles was determined from a millimolar absorbancy change of 4.0 for 4-*N*-HO-Cyd and 4-*N*-HO-dCyd, or 2.0 for cytidine and deoxycytidine. One unit of activity was defined as the conversion of 1 μmole of substrate per min under the above reaction conditions.

Assay IV. A spectrophotometric assay was used to follow NADPH-linked hydroxylamine reductase activity of cell extracts by measuring the decrease in absorbance at 340 nm with time, as described by Lazzarini and Atkinson (19). The assay mixture contained hydroxylamine-hydrochloride, 2.0 μmoles; potassium pyrophosphate (*p*H 8.0), 32 μmoles; NADPH, 0.25 μmole; cell-free extract, 0.1 ml; and water to a final volume of 1.0 ml. Reactions were started by addition of cell extract, and the decrease in absorbancy at 30 C was followed with time. Hydroxylamine reductase activity was corrected for endogenous NADPH oxidation by subtracting the oxidation rate of a substrate-free control. The amount of hydroxylamine reduced (in micromoles) was determined by dividing the change in absorbancy at 340 nm (ΔA_{340}) by 6.22. One unit of hydroxylamine reductase activity was defined as 1 μmole of substrate reduced per min under assay conditions. All of the above assays were based on initial velocity measurements.

Chemical assays. Protein was determined by the method of Lowry et al. (21), with crystalline bovine serum albumin as a standard. In more purified preparations, protein was estimated by the $A_{280/260}$ method of Warburg and Christian (31). Similar results were obtained by both methods.

In studies on the stoichiometry of 4-*N*-HO-Cyd conversion to product with modified assay II, portions of the reaction mixture deproteinized with perchloric acid were assayed for both hydroxylamine and total ninhydrin-positive material. Hydroxylamine was estimated in triplicate samples with Nessler's reagent by the method of Fishbein (8). The substrate did not interfere with the assay at the levels used. Ninhydrin-positive material was determined by the method of Fels and Veatch (7) but the A_{570} was measured directly on the reaction mixture without dilution in propanol-water (1:1). Both ammonia and hydroxylamine gave equimolar color development, provided fresh reagent solutions were prepared daily (reference 8). Ammonia was determined in portions of samples deproteinized with trichloroacetic acid as described by Ressler (30). Neither hydroxylamine nor substrate interfered with normal color development at the levels employed in the reaction mixtures.

Chromatography. Ascending paper chromatography was carried out on Whatman 3MM paper in the following solvent systems: A, upper phase of water saturated *s*-butanol (5); B, diethylether-methanol-water-hydrochloric acid (50:30:15:4; reference 29). Descending paper chromatography was conducted on Whatman no. 1 paper in solvent C, isopropanol-hydrochloric acid-water (68:16.4:15.6; reference 32). Nucleosides and nucleotides were located on electrophoretograms and paper chromatograms with an ultraviolet lamp and cut out for quantitation. Their concentrations were determined spectrophotometrically after elution with 2 ml of 0.01 N HCl (22). The radioactivity in these samples was measured in a scintillation spectrometer (Nuclear-Chicago Corp.) with modified Bray's solution (3).

Purification of cytidine (deoxycytidine) deaminase. All steps in the purification of cytidine (deoxycytidine) deaminase (EC 3.5.4.5) were performed at 0 to 4 C. Forty grams of frozen *E. coli* B (ATCC 11303) grown to mid-log phase in a high peptone medium (General Biochemicals Inc., Chagrin Falls, Ohio) was thawed in 400 ml of 0.05 M Tris-hydrochloride (*p*H 8.0), and 60-ml portions were sonically oscillated for 3 min (Biosonik III, large probe). The sonically treated material was centrifuged at 15,000 × *g* for 30 min, and the residue was discarded.

After the supernatant fraction was diluted to 12 mg of protein per ml with 0.05 M Tris-hydrochloride (*p*H 8.0), 102 ml of 5% streptomycin sulfate was added dropwise to the crude extract (510 ml). The mixture was stirred for 15 min and then centrifuged at 15,000 × *g* for 20 min. The supernatant fraction was brought to 0.45 saturation with 131.5 g of ammonium sulfate, and the precipitate formed after 15 min of stirring was centrifuged and discarded. The level of ammonium sulfate in the supernatant fluid was raised to 0.75 saturation (97.0 g), and the precipitate that formed after 15 min of stirring was centrifuged and dissolved in 0.01 M Tris-hydrochloride (*p*H 8.0). The resultant solution was dialyzed overnight against two 2-liter changes of the same buffer.

The dialyzed extract (84 ml) was passed through a DEAE cellulose column (3.3 cm diameter by 9.0 cm long) that had been equilibrated first with 0.05 M Tris-hydrochloride (*p*H 8.0) and then with 0.01 M Tris-hydrochloride (*p*H 8.0). After the column was washed with 100 ml of 0.01 M Tris-hydrochloride (*p*H 8.0), the crude enzyme preparation was eluted in a stepwise manner with four 160-ml portions of the same buffer containing 0.1, 0.18, and 0.3 M NaCl, respectively. Enzyme activity, measured by assay III, began appearing at the end of the 0.18 M NaCl elution and was removed completely with the 0.3 M NaCl buffer. Fractions (10 ml) containing enzyme with a greater specific activity than that added to the column were pooled (250 ml), and the enzyme was precipitated by the addition of solid ammonium sulfate to 0.75 saturation (133.0 g). The mixture was stirred for 15 min and centrifuged. The precipitate was dissolved in 0.01 M Tris-hydrochloride (*p*H 8.0) and dialyzed overnight against two 2-liter changes of the same buffer.

The dialyzed enzyme (44 ml) was adsorbed to a second DEAE cellulose column (1.2 cm diameter by 18.0 cm long) prepared as described above. After the

column was washed with 50 ml of 0.01 M Tris-hydrochloride (*p*H 8.0), the enzyme was eluted with a linear gradient consisting of 250 ml of 0.01 M Tris-hydrochloride (*p*H 8.0), 0.5 M NaCl in the reservoir, and 250 ml of the same buffer with 0.15 M NaCl in the mixing chamber. Five-milliliter fractions were collected at a flow rate of 0.8 ml/min. Most of the enzyme activity was eluted from the column between 0.17 and 0.23 M NaCl, as determined by conductivity measurements. The fractions were pooled (103 ml), and the enzyme was precipitated by the addition of solid ammonium sulfate to 0.75 saturation (49.0 g). After 15 min, the precipitate was centrifuged, dissolved in a minimal volume of 0.01 M potassium phosphate buffer (*p*H 7.1), and dialyzed overnight against two 2-liter changes of the same buffer.

The dialyzed enzyme preparation (12.4 ml) was acidified to *p*H 5.1 by the dropwise addition of 1.0 N acetic acid. After the solution was stirred for 10 min, the resultant precipitate was removed by centrifugation at 3,000 × *g* for 10 min. The enzyme-containing supernatant fraction was decanted and immediately brought to neutrality by the dropwise addition of 1.0 N NH_4OH. The precipitate was washed with 3.0 ml of 0.01 M sodium acetate buffer (*p*H 5.0) and then centrifuged, and the supernatant fraction was decanted and neutralized as described above. The pooled supernatant fractions were dialyzed overnight against two 2-liter changes of 0.01 M potassium phosphate (*p*H 7.1), and the resultant dialysate (16.5 ml) was divided into several portions and stored at −10 C.

RESULTS

Growth studies with E. coli Bu⁻. Figure 1 gives the growth characteristics of Bu⁻ in minimal medium supplemented with different pyrimidines. It is seen that both 4-*N*-HO-dCyd (curve C, open circles) and 4-*N*-HO-Cyd (curve D, open circles) can supply the pyrimidine requirement for growth. When a starter culture grown on uracil was used as inoculum (Fig. 1), the generation time for growth on 4-*N*-HO-Cyd was 102 min, whereas that on all other pyrimidines tested was approximately 72 min. However, when a starter culture grown on 4-*N*-HO-Cyd was used as inoculum, the generation time for growth on all pyrimidines tested, including 4-*N*-HO-Cyd, was approximately 72 min (*data not shown*).

To demonstrate that 4-*N*-HO-Cyd supplied the total pyrimidine requirement for growth, Bu⁻ cells were grown in minimal medium supplemented with 4-*N*-HO-Cyd-2-^{14}C. After harvesting, the nucleic acids were extracted from the cells as previously described. Separation of RNA and DNA into nucleotides revealed that all of the radioactivity was associated with the pyrimidine fractions. Table 1 shows that the specific activity of the various pyrimidine species isolated from both RNA and DNA was almost identical with that of the 4-*N*-HO-Cyd-2-^{14}C supplement present initially in the medium.

Figure 1 also shows the effect of 0.8 mM tetrahydrouridine, a potent inhibitor of cytidine (deoxycytidine) deaminase activity (13), on the growth of Bu⁻. The drug had no effect in medium supplemented with cytosine, uracil, or uridine (curve A, open triangles) but caused a 90-min lag before logarithmic growth began in medium supplemented with cytidine (curve B, open triangles). In medium supplemented with 4-*N*-HO-dCyd or 4-*N*-HO-Cyd, the inhibitor produced an extension of the generation time from 72 to 96 min in the case of the former and from 102 to over 600 min in the case of the latter.

Effect of growth conditions on cellular levels of cytidine (deoxycytidine) deaminase and hydroxylamine reductase. An inoculum of 4-*N*-HO-Cyd-grown cells was used to grow liter cultures of Bu⁻ in minimal medium supplemented with various pyrimidine ribonucleosides. Tetrahydrouridine was included also in the case of cytidine. After growth to late log phase, the cultures were harvested, and extracts were prepared as previously described. The cytidine (deoxycytidine) deaminase and hydroxylamine reductase activities in the extracts were measured with assays I and IV, respectively, and are reported in Table 2. The level of cytidine (deoxycytidine) deaminase activity present in extracts of Bu⁻ supplemented with either uridine or cytidine was similar to that present in extracts from wild-type cells grown in unsupplemented medium. However, the addition of 0.1 mM tetrahydrouridine to Bu⁻ grown with a cytidine supplement resulted in a twofold increase in the cytidine (deoxycytidine) deaminase activity over that present in the inhibitor-free culture. Increasing the tetrahydrouridine level to 1.0 mM in the cytidine-supplemented wild-type cultures yielded cytidine (deoxycytidine) deaminase activities equivalent to those found in Bu⁻ grown in medium supplemented with 4-*N*-HO-Cyd.

Since hydroxylamine reductase might be indirectly involved in the utilization of 4-*N*-HO-Cyd by reducing it to cytidine, the activity of this enzyme was measured also.

Table 2 reveals that the level of NADPH-linked hydroxylamine reductase activity in crude extracts of both the wild-type and pyrimidine-requiring strains of bacteria remained constant. In Bu⁻, this enzyme was independent of pyrimidine growth supplement and the presence or absence of tetrahydrouridine.

Experiments with cell-free extracts. The radioactive product formed by the action of 0.01 M Tris-hydrochloride (*p*H 7.1) dialyzed extracts on 4-*N*-HO-Cyd-2-^{14}C was uridine, whereas that formed by the same extracts dialyzed against 0.01 M potassium phosphate (*p*H 7.1) was predomi-

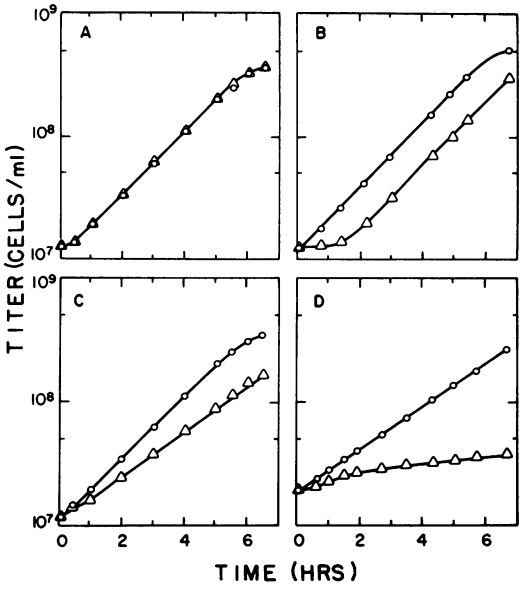

FIG. 1. *Growth of E. coli Bu⁻ in minimal medium containing various pyrimidine supplements (0.065 mM) in the presence and absence of tetrahydrouridine (0.8 mM). A, Cytosine, uracil, or uridine; B, cytidine; C, 4-N-hydroxy-deoxycytidine; D, 4-N-hydroxy-cytidine. Symbols: O, no inhibitor; Δ, plus tetrahydrouridine. The inoculum was grown in minimal medium supplemented with uracil.*

TABLE 1. *Specific radioactivities of pyrimidines isolated from the deoxyribonucleic acid and ribonucleic acid of Escherichia coli Bu⁻ grown in minimal medium supplemented with 4-N-hydroxy-cytidine-2-^{14}C*

Isolated pyrimidine	Specific activity (counts per min per μmole)[a]
Cytidine monophosphate	4.13×10^5
Uridine	4.03×10^5
Deoxycytidine monophosphate	4.05×10^5
Deoxythymidine monophosphate	3.88×10^5

[a] Specific activity of 4-N-hydroxy-cytidine-2-^{14}C added to medium was 4.07×10^5 counts per min per μmole.

nantly uracil with a trace of uridine. These results suggest that the initial product formed on the loss of hydroxylamine was uridine and that in the latter case uridine phosphorylase was responsible for the formation of uracil. In all cases, the ^{14}C-products recovered from paper chromatograms developed with solvent A had the same specific activity as the substrate.

Assay II was developed to follow more accurately the kinetics of interaction of 4-N-HO-Cyd with cell extracts. Under these assay conditions, substrate conversion appeared linear for about 30 min and then slowed continuously before reaching completion at about 180 min. Addition of different levels of tetrahydrouridine to a series of similar reactions, but terminated after 20 min, revealed that 2 μM inhibitor caused a 50% reduction in the rate of removal of 4-N-HO-Cyd in comparison with the inhibitor-free control. This level of tetrahydrouridine caused a similar reduction in the rate of cytidine-2-^{14}C deamination by the same extracts, when measured with assay I (*data not shown*).

Experiments with purified cytidine (deoxycytidine) deaminase. To determine whether cytidine (deoxycytidine) deaminase was directly involved in the conversion of 4-N-HO-Cyd to uridine, the enzyme was partially purified from *E. coli* B. A summary of the 185-fold purification is given in Table 3. Throughout the isolation procedure, the specific activities for the hydrolysis of 4-N-HO-Cyd, cytidine, and deoxycytidine, as measured with assay III, increased in a constant ratio of 0.02:1.0:2.5, respectively (Table 3).

The stoichiometry of 4-N-HO-Cyd hydrolysis was determined with the partially purified enzyme preparation in the modified assay II. At zero time and at 15-min intervals thereafter, 0.5-ml portions of the enzyme reaction were added to ice-cold acid (perchloric or trichloroacetic), and portions of the centrifuged solutions were assayed for quantities of substrate remaining, in addition to ammonia, hydroxylamine, and ninhydrin-positive material released. The results of a typical experiment are presented in Fig. 2, where it is shown that for every micromole of substrate enzymatically removed from the reaction mixture, 1 μmole of hydroxylamine and 1 μmole of ninhydrin-positive material were released. Am-

TABLE 2. *Cytidine (deoxycytidine) deaminase and hydroxylamine reductase levels in Escherichia coli B and Bu⁻ under different growth conditions*

Extract origin	Pyrimidine supplement	Tetrahydrouridine (mM)	Deaminase activity[a]	Reductase activity[b]
E. coli B	None	0	0.045	0.0102
E. coli Bu⁻[c]	Uridine	0	0.037	
	Cytidine	0	0.043	0.0120
		0.1	0.107	0.0113
		1.0	0.138	0.0101
	4-N-HO-Cyd	0	0.135	0.0110

[a] Determined at 37 C by assay I described in Materials and Methods; values expressed as units per milligram of protein.

[b] Determined at 30 C by assay IV described in Materials and Methods; values expressed as units per milligram of protein.

[c] Inoculum grown in minimal medium supplemented with 4-N-hydroxy-cytidine (4-N-HO-Cyd).

TABLE 3. *Purification of cytidine (deoxycytidine) deaminase from Escherichia coli B*

Step	Preparation	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)[a]	Purification (-fold)	Relative activities[b]
1	Sonic extract	6,100	330	0.054		0.02:1.0:2.5
2	Streptomycin sulfate	3,200	348	0.108	2	0.019:1.0:2.6
3	Ammonium sulfate	1,520	323	0.212	3.9	0.019:1.0:2.6
4	DEAE cellulose I	550	246	0.45	8.4	0.024:1.0:2.5
5	DEAE cellulose II	157	195	1.24	23	0.021:1.0:2.7
6	*p*H 5 precipitation	17.1	169	9.9	185	0.02:1.0:2.5

[a] Determined with cytidine as the substrate by assay III described in Materials and Methods.
[b] Rate of 4-*N*-hydroxy-cytidine:cytidine:deoxycytidine hydrolysis as determined by assay III.

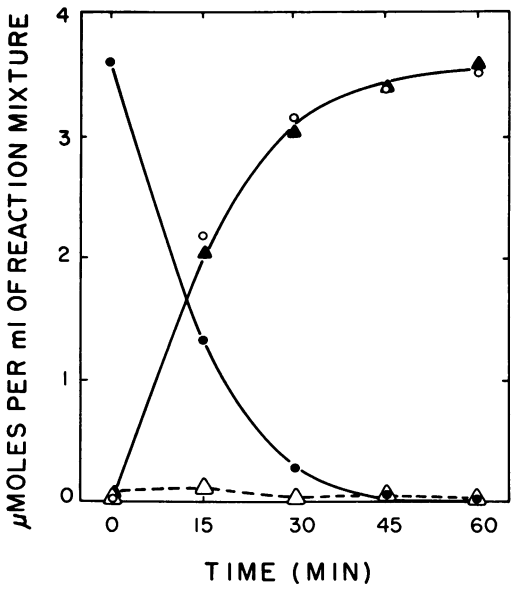

FIG. 2. *Stoichiometry of 4-N-hydroxy-cytidine hydrolysis by partially purified cytidine (deoxycytidine) deaminase measured with modified assay II. Symbols: ●, 4-N-HO-Cyd remaining; ○, hydroxylamine released; ▲, ninhydrin-positive material released; △, ammonia released.*

monia was not a detectable product of the reaction.

Hydroxylamine was identified also in samples of the reaction that had gone to completion by spotting perchloric acid-deproteinized samples on paper chromatograms and developing in solvent system B. In this system, 4-*N*-HO-Cyd and uridine migrated with R_F values of 0.42 and 0.62, respectively, in comparison with hydroxylamine with an R_F of 0.46. Hydroxylamine was easily distinguished from 4-*N*-HO-Cyd, since it turned orange-brown on spraying with picryl chloride (29) in comparison with 4-*N*-HO-Cyd which produced a bright orange color.

The activity of the purified enzyme preparation with cytidine, deoxycytidine, and 4-*N*-HO-Cyd as substrates (assay III) was measured from *p*H 5.6 to 10.0, with a wide range buffer consisting of 0.05 M each in boric acid, monosodium phosphate, and sodium acetate (12). The *p*H versus activity profiles (Fig. 3) obtained with either cytidine or deoxycytidine as the substrate revealed a single optimum at *p*H 7.1 with at least 80% of the maximum activity remaining at the *p*H extremes. With 4-*N*-HO-Cyd as the substrate, results similar to that for cytidine and deoxycytidine from *p*H 5.6 to a maximum at *p*H 7.1 were obtained. Then, as the *p*H was increased, the activity fell to 60% of the maximum at *p*H 7.4 and progressively diminished until at *p*H 10 only 10% remained (Fig. 3). When Tris-hydrochloride was used as the buffer, little activity was detected at *p*H 8.0 with 4-*N*-HO-Cyd as the substrate.

The K_m and V_{max} values for cytidine, deoxycytidine, 4-*N*-HO-Cyd, and 4-*N*-HO-dCyd were determined with the purified cytidine (deoxycytidine) deaminase preparation. Lineweaver-Burk plots (20) of the data obtained with assay III yielded the kinetic constants presented in Table 4. Although the 4-*N*-hydroxy derivatives are much poorer substrates than their corresponding amino analogues, the enzyme is more reactive with the respective deoxyribose-containing nucleosides.

Growth of E. coli OK 305. Figure 4 shows the growth characteristics of strain OK 305 on minimal medium containing various pyrimidine supplements. The generation time for OK 305 on medium supplemented with uridine or uracil was 72 min, whereas that supplemented with cytidine was 96 min. Figure 4 demonstrates that medium supplemented with either deoxycytidine, 4-*N*-HO-Cyd, or 4-*N*-HO-dCyd failed to support significant growth of OK 305 during the 7-hr incubation period. These data emphasize the importance of the deaminase for the utilization of the 4-*N*-HO-derivatives.

Although the inability of deoxycytidine to sup-

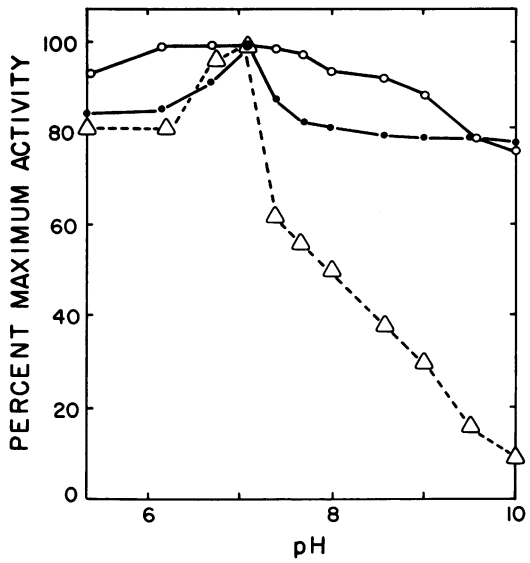

FIG. 3. *Effect of pH on the hydrolysis of substrates by partially purified cytidine (deoxycytidine) deaminase. Symbols:* O, *cytidine;* ●, *deoxycytidine;* Δ, *4-N-hydroxy-cytidine.*

TABLE 4. *Kinetic constants*[a] *for various substrates of cytidine (deoxycytidine) deaminase partially purified from Escherichia coli B*

Substrate	K_m (mM)	V_{max} (μmoles per min per mg of protein)
Deoxycytidine	0.052	25.2
Cytidine	0.095	9.9
4-*N*-hydroxy-deoxycytidine	0.059	0.60
4-*N*-hydroxy-cytidine	0.450	0.25

[a] Determined from Lineweaver-Burk plots obtained with assay III described in Materials and Methods.

port growth of OK 305 appears to contradict the deaminase studies that revealed deoxycytidine to be a better substrate than cytidine (Table 4), potential explanations for this finding are available. Karlström and Larsson (17) noted that, although OK 305 contained less than 0.02% of the wild-type ability to deaminate deoxycytidine, 1.5% of the cytidine-deaminating capacity remained. It was not determined whether the enzyme present differed from the wild-type enzyme in substrate specificity. An alternate explanation can be drawn from the studies of O'Donovan et al. (27), demonstrating the presence of a deoxycytidine triphosphate deaminating enzyme in *E. coli*. Since deoxycytidine cannot be phosphorylated, whereas cytidine can (28), the work of O'Donovan et al. (27) provides an alternate pathway for the conversion of cytidine to deoxyuridine and uracil:

cytidine → cytidine monophosphate →
cytidine diphosphate →
deoxycytidine diphosphate →
deoxycytidine triphosphate →
uracil
↑
deoxyuridine triphosphate → → deoxyuridine

Evidence favoring the latter possibility comes from the finding that OK 305 will grow in the presence of cytidine and tetrahydrouridine (1:10) but at a rate which is 70% of that found with cytidine alone (*data not shown*).

DISCUSSION

Growth experiments with *E. coli* Bu⁻ indicated that 4-*N*-HO-dCyd and 4-*N*-HO-Cyd could supply the total pyrimidine requirement necessary for the growth of this organism (Fig. 1). This fact was verified by the demonstration that the specific radioactivities of the pyrimidines isolated from the DNA and RNA of *E. coli* Bu⁻ cells grown in medium containing 4-*N*-HO-Cyd-2-^{14}C as the sole pyrimidine source were identical to that of the 4-*N*-HO-Cyd-2-^{14}C (Table 1).

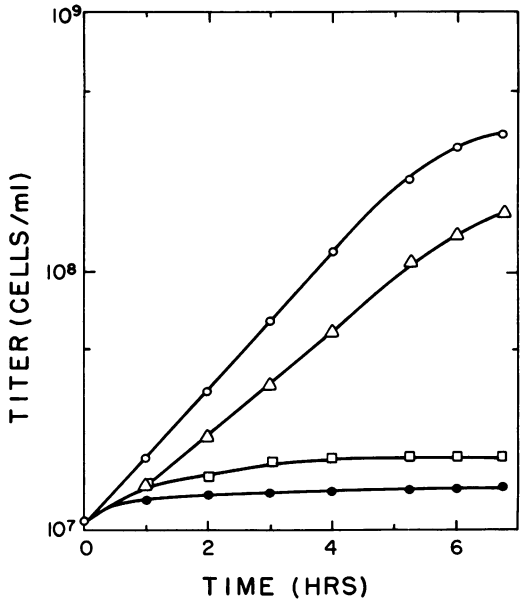

FIG. 4. *Growth of E. coli OK 305 in minimal medium containing various pyrimidine supplements at a final concentration of 0.065 mM. Symbols:* O, *uridine or uracil;* Δ, *cytidine;* □, *deoxycytidine or 4-N-hydroxy-deoxycytidine;* ●, *4-N-hydroxy-cytidine. The inoculum was grown in minimal medium supplemented with uracil.*

Since the 4-*N*-hydroxy compounds were not incorporated as such into RNA and DNA, but were first converted to cytosine- and uracil-containing nucleotides, two potential pathways for the utilization of these compounds were suggested: (i) the reduction to an amino nucleoside via a hydroxylamine reductase-type reaction, followed by deamination, or (ii) the direct elimination of hydroxylamine from the 4-*N*-hydroxy derivative.

Several lines of evidence suggested that cytidine (deoxycytidine) deaminase might be involved in the metabolism of the 4-*N*-hydroxy derivatives. First, tetrahydrouridine did not affect *E. coli* Bu⁻ growth on any of the pyrimidines tested with the exception of cytidine, 4-*N*-HO-Cyd, and 4-*N*-HO-dCyd (Fig. 1). The inhibition of growth on cytidine was transient, and the normal generation time was restored after about 2 hr; however, the inhibition of growth on the 4-*N*-hydroxy derivatives continued throughout the incubation period. Second, extended growth of *E. coli* Bu⁻ on 4-*N*-HO-Cyd yielded a threefold increase in the normal cellular levels of cytidine (deoxycytidine) deaminase (Table 2). This increase was coincident with the decrease in the generation time of *E. coli* Bu⁻ from 96 to 72 min noted when cells were grown for extended periods in the presence of 4-*N*-HO-Cyd. Finally, the conversion of both 4-*N*-HO-Cyd and cytidine to uridine by cell-free extracts was inhibited to about the same extent by similar concentrations of tetrahydrouridine.

Addition of tetrahydrouridine to Bu⁻ cells growing on cytidine also resulted in a threefold increase in the cellular levels of cytidine (deoxycytidine) deaminase activity (Table 2), a result that may explain the transient nature of the inhibition of Bu⁻ cells growth on cytidine by tetrahydrouridine (Fig. 1). The inability of Bu⁻ cells to overcome the inhibition by tetrahydrouridine when grown on the 4-*N*-hydroxy derivatives, even with a threefold increase in cytidine (deoxycytidine) deaminase, is apparently the result of the slow rate at which these compounds are utilized in comparison with cytidine. *E. coli* OK 305, which contains about 1.5% of the wild-type cytidine (deoxycytidine) deaminase activity (17), has a generation time on cytidine of 96 min (Fig. 4) which is the same as that for Bu⁻ growing on 4-*N*-HO-Cyd (Fig. 1). With cell-free extracts from Bu⁻ and with the partially purified cytidine (deoxycytidine) deaminase preparation from *E. coli* B (Table 4), 4-*N*-HO-Cyd and 4-*N*-HO-dCyd hydrolysis proceeded at about 2 and 6%, respectively, of that for cytidine. This conversion correlates well with the above mentioned growth rates and indicates that the prolonged generation time of 96 min in comparison with the normal of 72 min results from the rate-limiting conversion of cytidine by OK 305 cells and of 4-*N*-HO-Cyd by Bu⁻ cells.

Additional confirmation for the involvement of cytidine (deoxycytidine) deaminase in the metabolism of the 4-*N*-hydroxy nucleosides is provided by the finding that these compounds cannot satisfy the pyrimidine requirement of *E. coli* OK 305 (Fig. 4).

The most convincing evidence, however, for the involvement of the cytidine (deoxycytidine) deaminase comes from the demonstration of the direct conversion of the 4-*N*-HO-Cyd to hydroxylamine and uridine by a partially purified preparation of this enzyme (Fig. 2), and thus appears to rule out a hydroxylamine reductase-type reaction. Since the hydroxylamine reductase activity measured in a given extract was from 1.5 to 10 times greater than that involved in the release of hydroxylamine from either of the 4-*N*-hydroxy compounds (Table 2), little or no hydroxylamine should accumulate in the cell. This computation was made with the V_{max} values in Table 4. The actual level of hydroxylamine reductase activity was probably greater than quoted since it was measured at 30 C, whereas the cytidine (deoxycytidine) deaminase activity was measured at 37 C. This reductive process serves to protect the organism from the toxic effects of hydroxylamine, particularly those involved in the initiation of protein synthesis (18, 26).

The direct elimination of hydroxylamine by the *E. coli* deaminase appears to be a unique property of this enzyme, since a similar reaction is not encountered with deoxycytidylate deaminase and 4-*N*-HO-dCMP, although the latter is an effective inhibitor of the enzyme. Whether chick embryo mince utilizes 4-*N*-HO-dCyd (22), as does *E. coli*, remains to be seen.

Although studies with the cytidine (deoxycytidine) deaminases from *Bacillus cereus* (16) and bakers' yeast (15) indicate that these enzymes are allosterically regulated, similar studies with the *E. coli* deaminase failed to demonstrate any allosteric effect. The enzyme from *B. cereus* was inhibited by xanthosine monophosphate, quanosine triphosphate, cytidine monophosphate, quanosine monophosphate, and cytidine triphosphate (16), and the bakers' yeast enzyme was inhibited by cytidine monophosphate (15). By using assay III, the partially purified cytidine (deoxycytidine) deaminase from *E. coli* was tested for inhibition by 0.2 and 0.5 mM concentrations of the following nucleotides; cytidine monophosphate, cytidine triphosphate, quanosine monophosphate, quanosine triphosphate, uridine monophosphate, ATP, deoxycytidine

monophosphate, deoxycytidine triphosphate, deoxythymidine monophosphate, deoxythymidine triphosphate, deoxyquanosine monophosphate, deoxyquanosine triphosphate, deoxyadenosine monophosphate, deoxyuridine monophosphate. None of the nucleotides tested had a significant effect on the activity of *E. coli* cytidine (deoxycytidine) deaminase.

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